The microRNAs control eyelid development through regulating Wnt signaling

Takahiro Nagai[1,2], Supaluk Trakanant[1], Maiko Kawasaki[1,3], Katsushige Kawasaki[1,3,4], Yurie Yamada[1,4], Momoko Watanabe[1], James Blackburn[3], Yoko Otsuka-Tanaka[3,5], Mitsue Hishinuma[5], Atsushi Kitatmura[2], Fumiya Meguro[1], Akane Yamada[1,2], Yasumitsu Kodama[2], Takeyasu Maeda[4, 6], Qiliang Zhou[7], Yasuo Saijo[7], Akihiro Yasue[8], Paul T. Sharpe[3], Robert Hindges[9], Ritsuo Takagi[2] and Atsushi Ohazama[1,3,*]

1: Division of Oral Anatomy, Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
2: Division of Oral and Maxillofacial Surgery, Department of Health Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
3: Department of Craniofacial Development and Stem Cell Biology, Dental Institute, Kings College London, London. United Kingdom
4: Oral Life Science, Research Center for Advanced Oral Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
5: Department of Special Needs Dentistry, Nihon University School of Dentistry at Matsudo, Matsudo, Japan
6: Faculty of Dental Medicine, University of Airlangga, Surabaya 60132, Indonesia
7: Department of Medical Oncology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.
8: Department of Orthodontics and Dentofacial Orthopedics, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima City, Tokushima, Japan.
9: MRC Centre for Developmental Neurobiology, King's College London, New Hunt's House, Guy's Campus, London SE1 1UL, UK

*: Corresponding author: Atsushi Ohazama
Division of Oral Anatomy,
Department of Oral Biological Science,
Niigata University
Graduate School of Medical and Dental Sciences,
2-5274, Gakkocho-dori, Chuo-ku,
Niigata 951-8514, Japan
Tel: +81-25-227-2816
Fax: +81-25-223-6499
E-mail: atsushiohazama@dent.niigata-u.ac.jp

Running title; miRNAs in eyelid development

Key words: Eyelid development; MicroRNA; Dicer; mesenchyme; Wnt, Shh, Fgf, Bmp

Manuscript information; Text Pages = 25
Figures = 7
Supplementary Table = 1
Word and character counts: Words in paper = 7122

Words in Abstracts = 182
Characters in title (including spaces) = 73

ABSTRACT

Background: The timing, location, and level of gene expression are crucial for normal organ development, since morphogenesis requires strict genetic control. MicroRNAs (miRNAs) are noncoding small single-stranded RNAs that play a critical role in regulating gene expression level. Although miRNAs are known to be involved in many biological events, the role of miRNAs in organogenesis is not fully understood. Mammalian eyelids fuse and separate during development and growth. In mice, failure of this process results in the eye-open at birth (EOB) phenotype. **Results:** It has been shown that conditional deletion of mesenchymal *Dicer* (an essential protein for miRNA processing; *Dicer*$^{f/f}$;*Wnt1Cre*) leads to the EOB phenotype with full penetrance. Here, we identified that the upregulation of Wnt signaling resulted in the EOB phenotype in *Dicer* mutants. Downregulation of Fgf signaling observed in *Dicer* mutants was caused by an inverse relationship between Fgf and Wnt signaling. Shh and Bmp signaling were downregulated as the secondary effects in *Dicer*$^{f/f}$;*Wnt1Cre* mice. Wnt, Shh and Fgf signaling were also found to mediate the epithelial–mesenchymal interactions in eyelid development. **Conclusion:** miRNAs control eyelid development through Wnt signaling.

INTRODUCTION

MicroRNAs (miRNAs) are 19–25nt noncoding small single-stranded RNAs that negatively regulate gene expression by binding to target mRNAs. This binding is sequence specific and impairs the translation and stability of target mRNAs (Berezikov et al., 2006, Xie et al., 2005). miRNAs are thus crucial molecules for regulating precise gene expression. Morphogenesis including organogenesis is composed of many sequential processes, which requires strict genetic control. Hence, the timing, location, and level of gene expression should be highly accurate for normal organ development. Therefore, it is believed that miRNAs play a critical role in organ development. However, the role of miRNAs in organogenesis is not fully understood. Proper experimental model is required to explore the precise role of miRNAs in organogenesis.

The eyelid is an important organ for protecting and lubricating the surface of the eye. Each blink serves to spread fresh tears, remove debris, and replenish the smooth optical surface of the eye. Eyelids are constituted by many components including connective tissue, muscle, glands, and epithelium. Therefore, cells derived from all three germ layers are involved in eyelid formation. Eyelid formation also requires epithelial–mesenchymal interaction (Rubinstein et al., 2016, Ohuchi 2012). The epithelium is divided into the palpebral epidermis (the outer surface of the eyelid) and the palpebral conjunctiva (the inner surface of the eyelid). The palpebral conjunctiva is continuous with the bulbar conjunctiva (ocular conjunctiva), which covers the globe (Fig. 1A). The superficial bulbar conjunctival epithelium is continuous with the corneal epithelium (the outermost layer of the cornea).

Mammalian eyes require temporary fusion between the upper and lower eyelids during development and growth, which is believed to be essential events for correct eye development. The timing of eyelid fusion and separation is different between species. Murine eyelid development begins by groove formation at embryonic day 11 (E11), with protrusion of the eyelid primordia occurring at E12-E13. Epithelial ridges at the tip of the eyelid primordia meet and fuse to form an epithelial bridge at E15-E16 (Fig. 1B). The eyelids remain fused until postnatal day 12 when eyelid separation occurs (Findlater et al., 1993). Failure of these processes results in the eye-open at birth (EOB) phenotype in mice. Unlike the mouse, human eyelids fuse and re-open *in utero* (Byun et al., 2011).

miRNAs are synthesized in the cell by a multiple-step process and are initially transcribed as long primary transcripts by RNA polymerase II (Maiorano et al., 2012). The first transcript (pri-RNA) folds into a characteristic hairpin, which is cleaved by Drosha in complex with DGCR8 to release the 60nt stem-loop precursors (pre-miRNAs) that are exported into the cytoplasm. The cytoplasmic pre-miRNAs undergo final cleavage by the ribonuclease, Dicer, to produce mature miRNAs that assemble into an RNA-induced silencing complex (RISC). miRNAs are known to play critical roles in many biological events (Liu et al., 2015; Peng et al., 2013; Gargalionis et al., 2013, Otsuka-Tanaka et al., 2013, Oommen et al., 2012). Previous studies have shown an EOB phenotype in mice with mesenchymal conditional deletion of *Dicer*, but the molecular mechanisms remain unclear (Nie et al., 2011; Zehir et al., 2010; Huang et al., 2010, Sheehy et al., 2010). The EOB phenotype is an excellent model for understanding the precise role of miRNAs in organogenesis, since the EOB

phenotype has a readily identifiable morphology and was observed with full penetrance in *Dicer* mutant mice.

In this study, a detailed analysis of eyelid development was performed in mice with mesenchymal conditional deletion of *Dicer*. Our results indicate that the lack of miRNAs leads to the upregulation of Wnt signaling, which results in the EOB phenotype.

RESULTS

The eyelid develops through direct interaction between the epithelium and mesenchyme. We found that many miRNAs were expressed in both tissue types of the eyelid primordia (Fig. 1C–1J). Since mesenchymal deletion of *Dicer* is known to lead to the EOB phenotype, miRNAs in the mesenchyme are essential for eyelid development (Nie et al., 2011; Zehir et al., 2010; Huang et al., 2010, Sheehy et al., 2010). However, it remains unclear whether miRNAs in the epithelium also play a critical role in eyelid formation. To address this question, we generated mice with an epithelial conditional deletion of *Dicer* using the *keratin-14* (*K14*) promotor (*Dicer*$^{fl/fl}$;*K14Cre*). *K14* expression is observed in the eyelid epithelium from early stages of eye development (Fig. 2A, N=14/14, data not shown). Unlike mesenchymal deletion of miRNAs, no EOB phenotype could be detected in *Dicer*$^{fl/fl}$;*K14Cre* mice, although no *Dicer* expression was confirmed in the eyelid epithelium of *Dicer*$^{fl/fl}$;*K14Cre* mice (Fig. 2C, 2D). These results suggest that mRNA under the control of miRNAs in the eyelid epithelium are not crucial for eyelid development.

As previous papers revealed, we confirmed that mice with a mesenchymal conditional deletion of *Dicer* by *Wnt1Cre* mice (*Dicer*$^{fl/fl}$;*Wnt1Cre*) exhibit the EOB phenotype with full penetrance (N=45/45; Fig. 3B; Nie et al., 2011; Zehir et al., 2010; Huang et al., 2010, Sheehy et al., 2010). To assess whether this phenotype is caused by premature opening of the eye or an earlier developmental defect in the tissue fusion, we examined *Dicer*$^{fl/fl}$;*Wnt1Cre* mice at different embryonic stages. Initiation of the eyelid primordium is observed as a groove formation in wild-type embryos at E11.5-E12.5, which was

indistinguishable in *Dicer* mutant mice (Fig. 3C, 3D; data not shown). Eyelid primordia then protrude in wild-type mice at E12.5-E13.5 but were retarded in *Dicer* mutants (data not shown). The epithelial ridge of eyelid primordia was fused in wild-type mice around E15.5, but an epithelial ridge could not be detected in *Dicer* mutants at this stage (Fig. 3E, 3F). These results indicate that the EOB phenotype was caused by an arrest of eyelid development and not premature eyelid opening. LacZ-positive cells were present in the entire mesenchyme of the eyelid primordium in *R26R*^{lacZ};*Wnt1Cre* mouse embryos, indicating that the mesenchyme of eyelid primordia is mostly composed of neural crest-derived cells when eyelid primordia initiate and protrude (Fig. 3G). This suggests that miRNAs are removed from most eyelid mesenchyme at the stage in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice. In order to confirm the deletion of matured miRNAs in *Dicer* mutant eyelid mesenchyme, semiquantitative reverse transcription-PCR analysis was performed. miRNAs observed in wild-type eyelid mesenchyme confirmed by *in situ* hybridazation were absent in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice (Fig. 3H).

Eyelid development is known to be under the control of an intricate association of signaling pathways such as Shh, Bmp, Fgf, and Wnt (Gage et al., 2008; Luetteke et al., 1993, 1994; Li et al., 2001; Vassalli et al., 1994; Tao et al., 2005; Huang et al., 2009; Mine et al., 2005; Zhang et al., 2003, Zenz et al., 2003, Weston et al., 2004, Takatori et al., 2008, Smith et al., 2000, Mine et al., 2005, Li et al., 2003). To identify candidate signaling pathways related to EOB phenotypes in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice, we performed qPCR, *in situ* hybridization, and immunohistochemistry analyses.

The Wnt signaling pathway is known to be involved in eyelid formation (Gage et al., 2008). To understand Wnt signaling activity in mutant eyelid primordia, *Axin2* and *Nkd1* (markers of Wnt signaling; Wharton et al., 2001, Lustig et al., 2002) was examined. We found upregulation of *Axin2* and *Nkd1* expression in *Dicer* mutants (Fig. 4B, 4C; P=0.043114). It has been shown that upregulation of Wnt signaling due to a *Dkk2* mutation leads to the EOB phenotype (Gage et al., 2008), as well as ectopic hair follicle formation in the palpebral conjunctiva and a lack of transdifferention of the palpebral conjunctiva into epidermal tissue (Gage et al., 2008). Close morphological analysis was therefore performed in *Dicer* mutant mice. Ectopic hair follicles were also found in the palpebral conjunctiva of *Dicer*$^{fl/fl}$;*Wnt1Cre* mice (Fig. 4E). To confirm whether the palpebral conjunctiva transdifferentiated into epidermis, we assessed the immunolocalization of Filaggrin (a differentiation marker of epidermis) in *Dicer* mutant mice. Filaggrin-positive cells could not be detected in the palpebral conjunctiva of *Dicer*$^{fl/fl}$;*Wnt1Cre* mice, suggesting that this tissue does not differentiate into epidermis (Fig. 4G). In addition to these phenotypes, loss of the characteristic wavy morphology of the cornea stroma has also been reported in *Dkk2* mutant mice (Gage et al., 2008). Keratocytes of the cornea stroma exhibit a characteristic wavy morphology in wild-type mice at E18.5, which has not not been present in *Dkk2* mutants (Gage et al., 2008). *Dicer*$^{fl/fl}$;*Wnt1Cre* mice also showed the lack of the wavy morphology of the cornea stroma (Fig. 4I). Thus, *Dicer* mutant mice exhibited ectopic hair formation without transdifferentiation of the palpebral conjunctiva into epidermis and loss of characteristic morphology of cornea stroma, which were phenocopies these of the *Dkk2* mutants. To confirm whether the upregulation of Wnt signaling is a direct cause of the EOB phenotype in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice, we cultured *Dicer* mutant heads with DKK2 protein

using the oxygenated rolling bottle culture system. This method of culture permits eyelid closure of wild-type mouse heads, whereas no eyelid formation was observed in *Dicer* mutants (N=15/15 WT, N=18/18 *Dicer* mutants; Fig. 4J–4O). The EOB phenotype of the *Dicer*$^{fl/fl}$;*Wnt1Cre* mice was completely rescued by DKK2 protein (N=5/5; Fig. 4P–4R). DKK1 protein was also found to rescue the EOB phenotype in *Dicer* mutants (N=3/3; data not shown). These data suggest that the upregulated Wnt signaling is directly linked to the EOB phenotype in the *Dicer*$^{fl/fl}$;*Wnt1Cre* mice.

qPCR analysis also showed the significant reduction of *Gli1* expression (a marker of Shh signaling) in eyelid mesenchyme of *Dicer*$^{fl/fl}$;*Wnt1Cre* mice (Fig. 5A; $P = 0.026867$). *Gli1* was found to be expressed in both the epithelium and mesenchyme of the eyelid primordia (Fig. 5B). This indicates that Shh signaling is activated in both eyelid epithelium and mesenchyme in wild-type mice. In order to understand the role of epithelial or mesenchymal Shh signaling in eyelid development, we generated mice with conditional deletion of *Smo* (an essential protein for Shh signaling activation) using *Wnt1Cre* (*Smo*$^{fl/fl}$;*Wnt1Cre*) or *K14Cre* (*Smo*$^{fl/fl}$;*K14Cre*) mice. However, neither *Smo*$^{fl/fl}$;*Wnt1Cre* nor *Smo*$^{fl/fl}$;*K14Cre* mice displayed the EOB phenotype (Fig. 5D, 5E). On the other hand, it has been shown that mice with conditional mutation of *Smo* using *Osr2Cre* mice possess an EOB phenotype (Lan and Jiang 2009). Unlike *Wnt1Cre*, which is expressed only in the mesenchyme, *Osr2* is found to be expressed in both epithelium and mesenchyme of wild-type eyelid primordia (Fig. 5F; Gao et al., 2009, Lan and Jiang 2009). Taken together, it is likely that lack of Shh signaling results in an EOB phenotype when Shh signaling is deleted from both epithelium and mesenchyme. To further investigate the effect of the downregulation of Shh signaling on the

EOB phenotype in *Dicer* mutants, we cultured *Dicer* mutant heads with SAG (a Smo agonist) to increase Shh signaling in *Dicer* mutant eyelid primordia using the oxygenated rolling bottle culture system. *Gli1* was confirmed to be upregulated in SAG treated *Dicer* mutants by qPCR (Fig. 5G; P=0.0358). We also injected SAG into mother mice to increase Shh signaling in *Dicer* mutant embryos at E12.5, E13.5, and E14.5 (20 μg/g body weight). However, no sign of rescue of the EOB phenotype could be detected in *Dicer* mutants with SAG (N=6/6 rolling bottle culture system with SAG, N=4/4 injection of SAG; Fig 5I, 5K, data not shown). These data indicate that the downregulation of Shh signaling is unlikely to be a direct cause of the EOB phenotype in *Dicer* mutant mice.

The Fgf signaling pathway is also known to regulate eyelid formation, since both *Fgf10* and *Fgfr2* mutations lead to the EOB phenotype (Li et al., 2001, Tao et al., 2005). To understand Fgf signaling activity in the *Dicer* mutant eyelid primordia, p-ERK (a marker of Fgf signaling) was examined in *Dicer* mutants. In wild-type mice, p-ERK positive cells were observed in the eyelid epithelium, which is consistent with *Fgfr2* expression (Fig. 6A, Tao et al., 2005). p-ERK positive cells were reduced in the *Dicer*$^{fl/fl}$;*Wnt1Cre* mice (Fig. 6B). *Fgf10* expression was also found to be reduced in *Dicer* mutant eyelid primordia, although statistical analysis showed no significant difference between wild-type and *Dicer* mutants (Fig. 6C). Unlike *Dicer* mutants, ectopic hair formation or loss of characteristic morphology of cornea stroma could not be detected in *Fgf10* mutant mice (Fig. 6E, 6G). To further investigate the effect of downregulating Fgf signaling on the EOB phenotype in *Dicer* mutant mice, we cultured *Dicer* mutant heads with FGF10 protein to activate Fgf signaling in *Dicer* mutant eyelid primordia using the oxygenated rolling bottle culture system.

However, no sign of rescue of the EOB phenotype could be detected in *Dicer* mutant heads with FGF10 proteins (N=4/4; Fig. 6H–6J). This suggests that changes in Fgf signaling are unlikely to be a direct cause of the EOB phenotype in *Dicer* mutant mice.

The Bmp signaling pathway is also known to be involved in eyelid formation (Huang et al., 2009). To understand Bmp signaling activity in mutant eyelid primordia, p-Smad1/5/9 (a marker of Bmp signaling) was examined. Subtle p-Smad1/5/9 immunolocalization was found only in the tip of eyelid primordia of wild-type mice at E12.5 and E13.5 (Fig. 7A, 7C). P-Smad1/5/9 positive cells were more pronounced in wild-type at E14.5 when eyelid primordia became more evident (Fig. 7E). In *Dicer* mutants at E12.5 and E13.5, p-Smad1/5/9 positive cells could not be detected, while obvious eyelid primordia was not formed (Fig. 7B, 7D). Subtle Smad1/5/9 immunolocalization was observed in *Dicer* mutant mice at E14.5 when the size of mutant eyelid primordia became similar to those of E12.5 wild-type eyelid (Fig. 7A, 7F). *Bmp4* has been shown to be involved in eyelid development (Huang et al., 2009). At E12.5, *Bmp4* expression was found to be reduced in *Dicer* mutant eyelid primordia, although statistical analysis showed no significant difference between wild-type and *Dicer* mutants (Fig. 7G). To further understand whether the reduced *Bmp4* expression is involved in the EOB phenotype in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice, we cultured *Dicer* mutant heads with BMP4 protein using the oxygenated rolling bottle culture system. However, no sign of rescue of the EOB phenotype could be observed in *Dicer* mutant with BMP4 proteins (N=4/4; Fig. 7H). This indicates that altered Bmp signaling is unlikely to be a direct cause of the EOB phenotype in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice.

DISCUSSION

Smad4 is essential for mediating several signaling pathways including Bmp and Tgf, and mice with ocular conditional mutation of *Smad4* (*Smad4*$^{fl/fl}$/*LeCre*) show an EOB phenotype (Huang et al., 2009). Alteration of Bmp signaling has been shown to cause an EOB phenotype in *Smad4*$^{fl/fl}$/*LeCre* mice. Bmp signaling thus play a critical role in eyelid development. In wild-type mice, Bmp signaling was found to be activated in the tip of eyelid primordia. At E12.5 and E13.5, *Dicer*$^{fl/fl}$;*Wnt1Cre* mice exhibited the downregulation of Bmp signaling, while they also showed no obvious eyelid formation. The downregulation of Bmp signaling in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice was thus likely to be caused by the lack of eyelid primordia. We also found reduced *Bmp4* expression in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice. However, exogenous BMP4 protein failed to rescue the EOB phenotype in *Dicer* mutants, suggesting that the EOB phenotype in *Dicer* mutant mice is thus unlikely to involve Bmp signaling.

Fgf signaling is crucial pathway for eyelid formation, since both *Fgfr2* and *Fgf10* mutants show an EOB phenotype (Li et al., 2001, Tao et al., 2005). Fgf signaling is known to be activated in the eyelid epithelium, whereas *Fgf10* is expressed in the mesenchyme. Thus, Fgf controls eyelid formation via epithelial–mesenchymal interaction. Our results show that Fgf signaling in eyelid epithelium is downregulated in mice with mesenchymal deletion of miRNA, which was accompanied with reduced *Fgf10* expression in eyelid mesenchyme. miRNAs in mesenchyme are thus involved in the epithelial–mesenchymal interaction in eyelid development. Moreover, double mutation of the Fgf inhibitors *Sprouty1* and *Sprouty2* leads to upregulation of Fgf signaling, which also results in an EOB phenotype, suggesting

that either lack or upregulation of Fgf signaling results in the EOB phenotype (Kuracha et al., 2013). Thus, fine-tuning of Fgf signaling is essential for proper eyelid development. Overactivation of Fgf signaling due to *Sprouty1/2* mutation has been shown to result in the downregulation of Wnt signaling in the eyelid primordia. Conversely, downregulation of Fgf signaling due to an *Fgfr2* mutation has been shown to lead to the upregulation of Wnt signaling in the eyelid primordia (Huang et al., 2009). Together, these findings indicate that there is an inverse relationship between Fgf and Wnt signaling in eyelid development. Interestingly, Wnt signaling in the eyelid primordia is mainly activated in the mesenchyme, whereas Fgf signaling is activated in the epithelium. The inverse relationship between Fgf and Wnt signaling also mediates the epithelial–mesenchymal interaction. In this study, exogenous FGF10 protein could not rescue the EOB phenotype in *Dicer* mutants, although *Fgf10* was downregulated in *Dicer* mutants. Characteristic morphological features (ectopic hair follicle formation, characteristic wavy morphology of the cornea stroma keratocytes, and absence of palpebral conjunctiva differentiation into epidermis) found in *Dicer* mutants could not be detected in *Fgf10* mutants. Therefore, the downregulation of Fgf signaling in *Dicer* mutants is caused as a secondary effect through the upregulation of Wnt signaling.

Our results suggest that Shh signaling is downregulated in *Dicer* mutant mice but is not a direct cause of the EOB phenotype. It has been shown that Shh signaling is reliant on Fgf signaling during eyelid development (Li et al., 2001; Tao et al., 2005, Kuracha et al., 2013). This raises the possibility that changes in Shh signaling activity in *Dicer*$^{fl/fl}$;*Wnt1Cre* mice are caused by disturbance of the interaction between Wnt and Fgf signaling.

We found upregulation of Wnt signaling in *Dicer* mutant eyelid primordia, which was accompanied with same morphological phenotypes (ectopic hair follicle formation, characteristic wavy morphology of the cornea stroma keratocytes, and absence of palpebral conjunctiva differentiation into epidermis) to those in mice with upregulation of Wnt signaling (Gage et al., 2008). In addition, reduced Wnt signaling by exogenous DKK protein rescued the EOB phenotype in *Dicer* mutants. These indicate that the EOB phenotype was caused by the upregulation of Wnt signaling in *Dicer* mutants. In common with Fgf signaling, fine-tuning of Wnt signaling is necessary for proper eyelid development, since it has been shown that downregulation of Wnt due to a *Tcf3* mutation also results in an EOB phenotype (Wu et al., 2012). This also supports our hypothesis of the inverse relationship between Fgf and Wnt signaling in eyelid development. The fine-tuning mechanisms also raises the possibility that in organ culture experiments presented in this study, the amount of growth factors provided in the culture medium, whether the concentration is comparable to the in vivo situation, and the half-lives of the factors, could all affect the outcomes. The absence of miRNAs directly leads to an increase in mRNA, as they negatively regulate gene expression by binding to target mRNAs. Proteins that activate Wnt signaling are likely to be increased in *Dicer* mutant eyelid primodia due to the lack of miRNAs. It has been shown that several Wnt ligands are expressed in eyelid development (Liu et al., 2003). To analyze which of miRNAs expressed in the eyelid primordia could bind to mRNA of Wnt ligands, we used three programs (MiRBase, miRDB and TargetScan). Several miRNAs were identified as having binding potential for Wnt ligands (Supplementary Table). However, further investigation is needed to identify the target genes of miRNAs involving the upregulation of Wnt signaling in *Dicer* mutant eyelid development. We could not exclude

the possibility that these targets are novel factors or factors which have not been shown to be related to Wnt signaling, since Wnt signaling could be activated by a numerous factors including non-linagd proteins.

In common with *Fgf10*, *Bmp4* was also slightly reduced in *Dicer* mutants. It has been reported that there is interaction between Fgf and Bmp signaling in eyelid development (Huang et al., 2009). However, ectopic hair follicle formation found in *Dicer* mutants has also been shown in *Smad4*$^{flox/flox}$/*LeCre* mice, but not in *Fgf10* mutant mice (Huang et al., 2009). It is possible that there is specific interaction between Bmp and Wnt signaling in eyelid development.

Human eyelids are known to fuse and re-open *in utero* (Byun et al., 2011). Disturbance of embryonic eyelid formation leads to ablepharon – an absence or severe underdevelopment of the eyelids – a key feature of several syndromes such as ablepharon-macrostomia syndrome and blepharophimosis–ptosis–epicanthus inversus syndrome (Kallish et al., 2011, Rohena et al., 2011, Crisponi et al., 2001, Uda et al., 2004). However, little is known as to how failure of eyelid fusion or separation is associated with human eye anomalies. Understanding the developmental failures in mice with an EOB phenotype sheds light not only on the role of miRNAs in organogenesis and/or the mechanism of eyelid morphogenesis but also on the role of eyelid fusion and re-opening during human development. Eyelids are known to be one of the most difficult organs to repair because of their unique anatomic and functional requirements. Understanding the molecular basis of eyelid formation in mutant mice with an EOB phenotype may provide insights into developing new strategies for

reconstruction/regeneration therapy for the eyelid.

CONCLUSION

Conditional deletion of mesenchymal *Dicer* leads to the EOB phenotype with full penetrance. We identified that the EOB phenotype was caused by the upregulation of Wnt signaling, which was accompanied by downregulation of Shh and Fgf signaling as secondary effects. miRNAs thus control eyelid development through Wnt signaling.

MATERIALS AND METHODS

Production and analysis of transgenic mice

Smo$^{fl/fl}$, *Dicer*$^{fl/fl}$, *Wnt1Cre*, *K14Cre* , *R26R*^{LacZ} and *Fgf10*$^{-/-}$ mice were produced as described by Jeong et al., (2004), Harfe (2005), Danielian et al. (1998), Yi et al. (2006), Soriano (1999) and Yasue et al., (2014), respectively. Embryonic day 0 (E0) was taken to be midnight prior to finding a vaginal plug.

In situ hybridisation

In situ hybridisation was carried out to detect mRNAs using [^{35}S]UTP-labeled riboprobes or to detect miRNAs using locked nucleic acid probes labeled with DIG as described previously (Ohazama et al., 2008; Oommen et al., 2012).

Immunohistochemistry

After deparaffinization, sections were treated with proteinase K and then incubated with antibodies to Phosphorylated Smad1, Smad5 and Smad8 (p-Smad1/5/9; Cell Signaling Technology), Filaggrin (BioLegend) or Phosphrylated-ERK (p-ERK; Santa Cruz). Tyramide signal amplification system was performed (Parkin Elmer Life Science) for detecting anti-p-Smad1/5/9 and anti-p-ERK antibody. Alexa488 was used for detecting anti-Filaggrin antibody.

β-galactosidase staining

β-galactosidase staining was carried out as described previously (Ohazama et al., 2008).

Quantitative-PCR (Q-PCR)

Q-PCR was performed using GoTaq qPCR Master Mix (Promega) with the carboxy-X-rhodamine (CXR) Dye and Rotor-Gen-Q (Qiagen) detection system. All samples were run in triplicate for each experiment, and relative transcript abundance was normalized to the amount of GAPDH. The epithelium and mesenchyme from eyelid primordia were isolated following incubation in a solution of Dispase (3 mg/ml) for 10–15 min at 37°C. After incubation, the tissues were mechanically separated and mRNA extracted with the RNeasy Mini Kit (Qiagen).

Eyelid culture

Eyelid culture was carried out as described previously with some modifications (Imai et al., 1996, 1998). The upper head including the eyelid region was separated from the fetal body. The upper head explants were immediately placed into a glass bottle containing culture media comprising 100% immediately centrifuged (IC) rat serum. IC rat serum was incubated at 56°C for 30 minutes to inactivate complement. DKK1 protein (R&D systems; 50ng/ml), DKK2 protein (R&D systems; 50ng/ml), SAG (Merck; 100nM), BMP4 (100ng/ml) or FGF10 (R&D systems; 50ng/ml) was added in serum for the EOB phenotype rescue experiments. The culture bottles were attached to a rotator drum and rotated at 14 rpm at 37°C while being continuously supplied with 5% O_2/ 95% CO_2 gas mixture. At the end of the culture period, explants were fixed with 4% PFA, and processed for histological and molecular examination.

Semiquantitative reverse transcription-PCR

The mesenchyme from eyelid primordia were isolated as descrived above. MiRNAs were purified from eyelid mesenchyme with the miRNeasy mini kit (Qiagen), and reverse transcribed with the miScript PCR system (Qiagen). Semiquantitative PCRs for mature mmu-miR-148a-3p, mmu-miR-218-5p, and mmu-miR-378a-3p were performed with the miScript PCR system (Qiagen), using the miScript Primer Assays (Qiagen) for all mature miRNAs for 30 cycles (Tm = 55 °C).

Statistical analysis

Excel Toukei (ver. 6.0) was used for statistical analysis, which was done with a two-tailed unpaired Student's t test and Mann–Whitney U test. $P<0.05$ was considered statistically significant.

REFERENCES

Berezikov E, Cuppen E, Plasterk RH. 2006. Approaches to microRNA discovery. Nat Genet 38 Suppl:S2-7.

Byun TH, Kim JT, Park HW, Kim WK. 2011. Timetable for upper eyelid development in staged human embryos and fetuses. Anat Rec (Hoboken). 294:789-96.

Chai Y, Jiang X, Ito Y, Bringas P Jr, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM. 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development. 127:1671-9.

Chen G, Tan CS, Teh BK, Lu J. 2011. Molecular mechanisms for synchronized transcription of three complement C1q subunit genes in dendritic cells and macrophages. J Biol Chem 286:34941-34950.

Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, Bisceglia L, Zelante L, Nagaraja R, Porcu S. et al. 2001. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat Genet 27:159–166.

Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. 1998. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol 8:1323-1326.

Findlater GS, McDougall RD, Kaufman MH. 1993. Eyelid development, fusion and subsequent reopening in the mouse. J Anat 183:121-129.

Gao Y, Lan Y, Ovitt CE, Jiang R. 2009. Functional equivalence of the zinc finger transcription factors Osr1 and Osr2 in mouse development. Dev Biol 328:200-209.

Gage PJ, Qian M, Wu D, Rosenberg KI. 2008. The canonical Wnt signaling antagonist DKK2 is an essential effector of PITX2 function during normal eye development. Dev Biol 317:310-234.

Gargalionis AN, Basdra EK. 2013. Insights in microRNAs biology. Curr Top Med Chem 13:1493-502.

Harfe BD. 2005. MicroRNAs in vertebrate development. Curr Opin Genet Dev 15:410-415.

Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. 2004. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell 118:517-528.

Huang J, Dattilo LK, Rajagopal R, Liu Y, Kaartinen V, Mishina Y, Deng CX, Umans L, Zwijsen A, Roberts AB, Beebe DC. 2009. FGF-regulated BMP signaling is required for eyelid closure and to specify conjunctival epithelial cell fate. Development 136:1741-1750.

Huang T, Liu Y, Huang M, Zhao X, Cheng L. 2010. Wnt1-cre-mediated conditional loss of Dicer results in malformation of the midbrain and cerebellum and failure of neural crest and dopaminergic differentiation in mice. J Mol Cell Biol 2:152-163.

Imai H, Osumi N, Eto K. 1998. Contribution of foregut endoderm to tooth initiation of mandibular incisor in rat embryos. Eur J Oral Sci 106(Suppl 1):19–23.

Imai H, Osumi-Yamashita N, Ninomiya Y, Eto K. 1996. Contribution of early-emigrating midbrain crest cells to the dental mesenchyme of mandibular molar teeth in rat embryos. Dev Biol 176:151–165.

Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP. 2004. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. Genes Dev 18:937-951.
Kallish S, McDonald-McGinn DM, van Haelst MM, Bartlett SP, Katowitz JA, Zackai EH. 2011. Ablepharon-Macrostomia syndrome--extension of the phenotype. Am J Med Genet A 155A:3060-3062.
Kuracha MR, Siefker E, Licht JD, Govindarajan V. 2013. Spry1 and Spry2 are necessary for eyelid closure. Dev Biol 383:227-238.
Lan Y, Jiang R. 2009. Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. Development. 136:1387-1396.
Li C, Guo H, Xu X, Weinberg W, Deng CX. 2001. Fibroblast growth factor receptor 2 (Fgfr2) plays an important role in eyelid and skin formation and patterning. Dev Dyn 222:471-483.
Li G, Gustafson-Brown C, Hanks SK, Nason K, Arbeit JM, Pogliano K, Wisdom RM, Johnson RS. 2003. c-Jun is essential fororganization of the epidermal leading edge. Dev Cell 4:865-877.
Luetteke NC, Qiu TH, Peiffer RL, Oliver P, Smithies O, Lee DC. 1993. TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell 73:263-278.
Luetteke NC, Phillips HK, Qiu TH, Copeland NG, Earp HS, Jenkins NA, Lee DC. 1994. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes Dev 8:399-413.
Liu N, Bassel-Duby R. 2015. Regulation of skeletal muscle development and disease by microRNAs. Results Probl Cell Differ 56:165-190.
Liu H, Mohamed O, Dufort D, Wallace VA. 2003. Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. Dev Dyn 227:323-334.
Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Clevers H, Schlag PM, Birchmeier W, Behrens J. 2002. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol 22:1184-1193.
Maiorano NA, Hindges R. 2012. Non-coding RNAs in retinal development. Int J Mol Sci. 13:558-578.
Mine N, Iwamoto R, Mekada E. 2005. HB-EGF promotes epithelial cell migration in eyelid development. Development. 132:4317-4326.
Nie X, Wang Q, Jiao K. 2011. Dicer activity in neural crest cells is essential for craniofacial organogenesis and pharyngeal arch artery morphogenesis. Mech Dev 128:200-207.
Ohazama A, Johnson EB, Ota MS, Choi HY, Porntaveetus T, Oommen S, Itoh N, Eto K, Gritli-Linde A, Herz J, Sharpe PT. 2008. Lrp4 modulates extracellular integration of cell signaling pathways in development. PLoS One 3:e4092.
Ohuchi H. 2012. Wakayama Symposium: Epithelial-mesenchymal interactions in eyelid development. Ocul Surf 10:212-216.
Oommen S, Francois M, Kawasaki M, Murrell M, Kawasaki K, Porntaveetus T, Ghafoor S, Young NJ, Okamatsu Y, McGrath J, Koopman P, Sharpe PT, Ohazama A. 2012. Cytoplasmic plaque formation in hemidesmosome development is dependent on SoxF

transcription factor function. PLoS One 7:e43857.
Oommen S, Otsuka-Tanaka Y, Imam N, Kawasaki M, Kawasaki K, Jalani-Ghazani F, Anderegg A, Awatramani R, Hindges R, Sharpe PT, Ohazama A. 2012. Distinct roles of microRNAs in epithelium and mesenchyme during tooth development. Dev Dyn 241:1465-1472.
Otsuka-Tanaka Y, Oommen S, Kawasaki M, Kawasaki K, Imam N, Jalani-Ghazani F, Hindges R, Sharpe PT, Ohazama A. 2013. Oral lining mucosa development depends on mesenchymal microRNAs. J Dent Res 92:229-234
Peng L, Li Y, Zhang L, Yu W. 2013. Moving RNA moves RNA forward. Sci China Life Sci 56:914-920.
Rohena L, Kuehn D, Marchegiani S, Higginson JD. 2011. Evidence for autosomal dominant inheritance of ablepharon-macrostomia syndrome. Am J Med Genet A 155A:850-854.
Rubinstein TJ, Weber AC, Traboulsi EI. 2016. Molecular biology and genetics of embryonic eyelid development. Ophthalmic Genet 37:252-259.
Sheehy NT, Cordes KR, White MP, Ivey KN, Srivastava D. 2010. The neural crest-enriched microRNA miR-452 regulates epithelial-mesenchymal signaling in the first pharyngeal arch. Development. 137:4307-4316.
Smith RS, Zabaleta A, Kume T, Savinova OV, Kidson SH, Martin JE, Nishimura DY, Alward WL, Hogan BL, John SW. 2000. Haploinsufficiency of the transcription factors FOXC1 and FOXC2 results in aberrant ocular development. Hum. Mol. Genet 9:1021-1032.
Soriano P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21: 70-71.
Takatori A, Geh E, Chen L, Zhang L, Meller J, Xia Y. 2008. Differential transmission of MEKK1 morphogenetic signals by JNK1 and JNK2. Development 135:23-32.
Tao H, Shimizu M, Kusumoto R, Ono K, Noji S, Ohuchi H. 2005. A dual role of FGF10 in proliferation and coordinated migration of epithelial leading edge cells during mouse eyelid development. Development. 132:3217-3230.
Uda M, Ottolenghi C, Crisponi L, Garcia JE, Deiana M, Kimber W, Forabosco A, Cao A, Schlessinger D, Pilia G. 2004. Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. Hum Mol Genet 13:1171-1181.
Vassalli A, Matzuk MM, Gardner HA, Lee KF, Jaenisch R. 1994. Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. Genes Dev 15:414-427.
Wu CI, Hoffman JA, Shy BR, Ford EM, Fuchs E, Nguyen H, Merrill BJ. 2012. Function of Wnt/ β -catenin in counteracting Tcf3 repression through the Tcf3- β -catenin interaction. Development 139:2118-2129.
Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M. 2005. Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. Nature 434:338-345.
Yasue A, Mitsui SN, Watanabe T, Sakuma T, Oyadomari S, Yamamoto T, Noji S, Mito T, Tanaka E. 2014. Highly efficient targeted mutagenesis in one-cell mouse embryos mediated by the TALEN and CRISPR/Cas systems. Sci Rep 16:5705.
Yi R, O'Carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovsky A, Fuchs E. 2006. Morphogenesis in skin is governed by discrete sets of differentially expressed

microRNAs. Nat Genet 38:356-362.
Zehir A, Hua LL, Maska EL, Morikawa Y, Cserjesi P. 2010. Dicer is required for survival of differentiating neural crest cells. Dev Biol 340:459-467.
Zhang L, Wang W, Hayashi Y, Jester JV, Birk DE, Gao M, Liu CY, Kao WW, Karin M, Xia Y. 2003. A role for MEK kinase 1 in TGF-beta/activin-induced epithelium movement and embryonic eyelid closure. EMBO J 22:4443-4454.
Zenz R, Scheuch H, Martin P, Frank C, Eferl R, Kenner L, Sibilia M, Wagner EF. 2003. c-Jun regulates eyelid closure and skin tumordevelopment through EGFR signaling. Dev Cell 4: 879-889.
Weston C.R. Wong A, Hall JP, Goad ME, Flavell RA, Davis RJ. 2004. The c-Jun NH2-terminal kinase is essential for epidermal growth factor expression during epidermal morphogenesis. Proc Natl Acad Sci USA 101: 14114-14119.
Wharton KA Jr, Zimmermann G, Rousset R, Scott MP. 2001. Vertebrate proteins related to Drosophila Naked Cuticle bind Dishevelled and antagonize Wnt signaling. Dev Biol 234:93-106.

Figure Legends

Figure 1. Structure of the eye and eyelid development.

(A) Schematic illustration of the components in the normal eye and eyelid. (B) Schematic illustration of eyelid development. (C-J) Frontal sections showing *in situ* hybridization of miRNAs at E14.5.

Figure 2. Eyelid development in *Dicer$^{fl/fl}$;K14Cre* mice

(A) Frontal sections showing *in situ* hybridization of *K14* in wild-type embryo at E14.5. (B, C) Frontal sections showing eyelids of wild-type mouse (B) and *Dicer$^{fl/fl}$;K14Cre* (C) at birth. (D) Q-PCR of *Dicer* on mRNA isolated from eyelid epithelium between wild-type and *Dicer$^{fl/fl}$;K14Cre* mice.

Figure 3. Eyelid development in *Dicer$^{fl/fl}$;Wnt1Cre* mice

(A, B) Newborn heads of wild-type mice (A) and the EOB phenotype in *Dicer$^{fl/fl}$;Wnt1Cre* (B). (C-F) Frontal sections showing the developing eyelids in the wild-type embryo (C, E) and *Dicer$^{fl/fl}$/Wnt1Cre* (D, F) at E12.5 (C, D) and E15.5 (E, F). (G) Frontal section showing LacZ staining in *R26R^{LacZ};Wnt1Cre* mice. (H) Semiquantitative RT-PCR for *miR148a*, *miR218* and *miR378a*.

Figure 4. Wnt signaling in *Dicer$^{fl/fl}$;Wnt1Cre* mice.

(A, B) Frontal sections showing *in situ* hybridization of *Axin2* in wild-type (A) and *Dicer$^{fl/fl}$;Wnt1Cre* (B) at E12.5. Arrowheads indicate gene expression. (C) Q-PCR of *Nkd1* on

mRNA isolated from eyelid mesenchyme between wild-type and *Dicer$^{fl/fl}$;Wnt1Cre* mice. *; $p<0.05$。 (D, E, H, I) Frontal sections showing palpebral conjuctiva (D, E) and cornea stroma (H, I) of wild-type (D, H) and *Dicer$^{fl/fl}$;Wnt1Cre* (E, I) embryos at E18.5. Arrow indicates ectopic hair follicles in the palpebral conjunctiva of *Dicer$^{fl/fl}$/Wnt1Cre* mice (E). (F, G) Frontal sections showing immunohistochemistry of Filaggrin in the developing eyelid at E14.5. Arrowheads indicate the palpebral conjunctiva. (J-R) Organ culture explants of wild-type (J-L), *Dicer* mutant alone (M-O) and *Dicer* mutant with DKK2 protein (P-R) before culture (J, M, P) and after 72 hours culture (K, L, N, O, Q, R). (L, O, R) Frontal sections showing histology of cultured explants. Arrowheads indicate developing eyelid in wild-type (L) and rescued eyelid in *Dicer* mutant (R).

Figure 5. Shh signal in eyelid development.

(A, G) Q-PCR of *Gli1* on mRNA isolated from eyelid primordia at E12.5. *; $p<0.05$。 (B, F) Frontal sections showing *in situ* hybridization of *Gli1* (B) and *Osr2* (F) in developing eyelid of wild-type at E12.5. Arrowheads indicate gene expression in developing eyelid. (C-E) Frontal sections showing newborn eyelids in wild-type (C), *Smo$^{fl/fl}$;Wnt1Cre* (D) and *Smo$^{fl/fl}$;K14Cre* (E). (H-K) Organ culture explants of wild-type (H, I) and *Dicer* mutant with SAG (J, K) before culture (H, J) and after 72 hours culture (I, K).

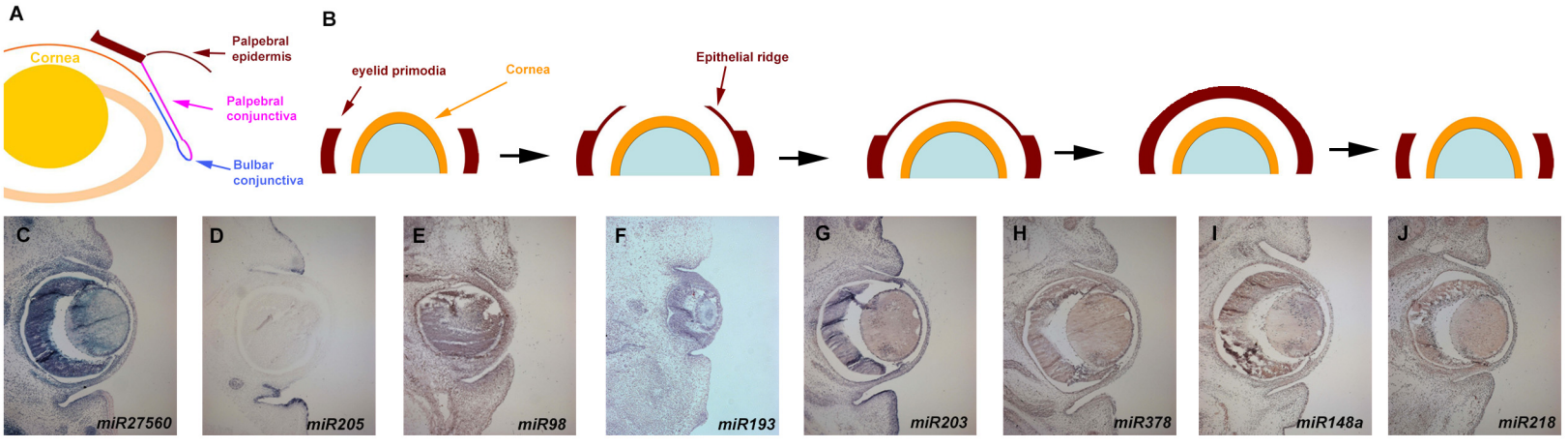
Figure 6. Fgf signaling in eyelid development.

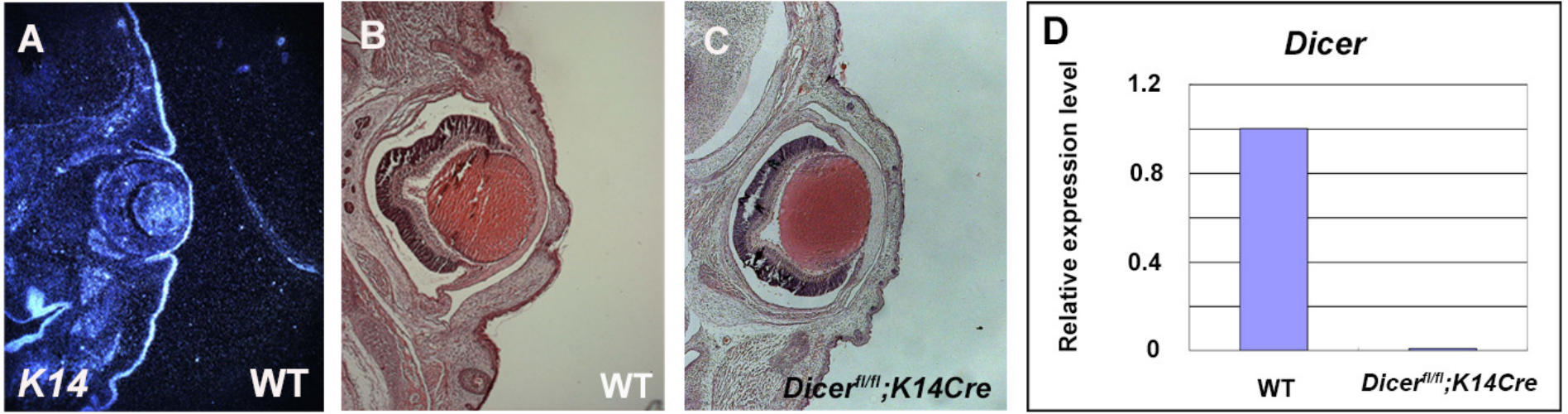
(A, B) Frontal sction showing immunohistochemistry of p-ERK. Arrowheads indicate p-ERK positive cells in wild-type (A) and *Dicer$^{fl/fl}$;Wnt1Cre* (B). (C) Q-PCR of *Fgf10* on mRNA isolated from eyelid primordia at E12.5. (D-G) Frontal sections showing the

palpebral conjunctiva (D, E) and cornea stroma (F, G) in wild-type (D, F) and *Fgf10*$^{-/-}$ (E, G) at E18.5. (H-J) Organ culture explants of *Dicer* mutant with FGF10 before culture (H) and after 72 hours culture (I, J). (J) Frontal section showing histology of cultured explants.

Figure 7. Bmp signaling in eyelid development.

(A-F) Frontal sections showing immunohistochemistry of p-Smad1/5/9 in wild-type (A, C, E) and *Dicer*$^{fl/fl}$*;Wnt1Cre* (B, D, F) at E12.5 (A, B), E13.5 (C, D) and E14.5 (E, F). Eyelid primordia including conjunctiva was outlined by red dots. Arrowheads indicate the tip of the eyelid primordia. (G) Q-PCR of *Bmp4* on mRNA isolated from eyelid mesenchyme between wild-type and *Dicer*$^{fl/fl}$*;Wnt1Cre* mice. (H-M) Organ culture explants of wild-type (H, I), *Dicer* mutant alone (J, K) and *Dicer* mutant with BMP4 protein (L, M) before culture (H, J, L) and after 72 hours culture (I, K, M).

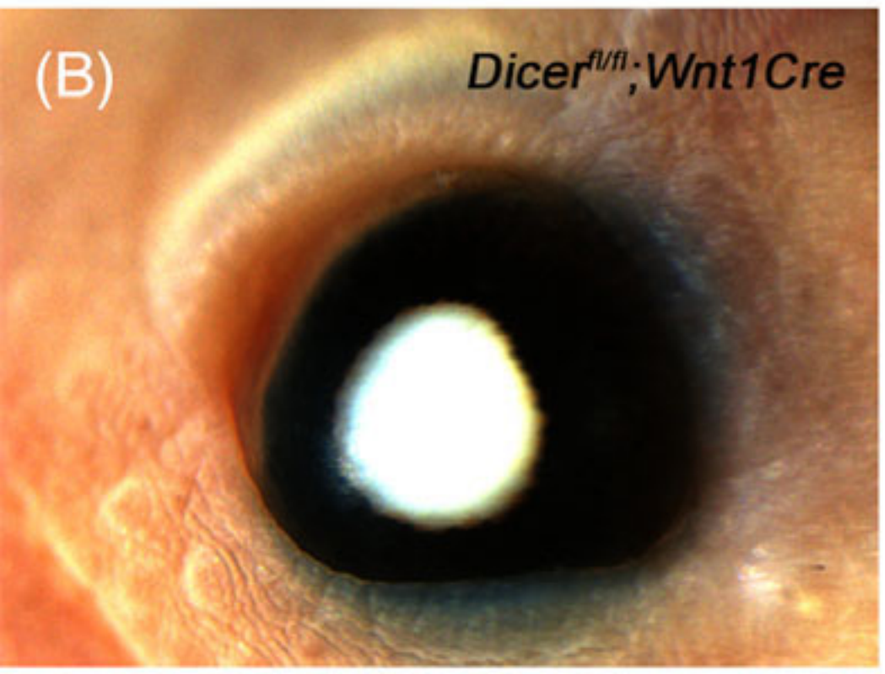

A
Cornea
Palpebral epidermis
Palpebral conjunctiva
Bulbar conjunctiva
B
eyelid primodia
Cornea
Epithelial ridge
C
miR27560
D
miR205
E
miR98
F
miR193
G
miR203
H
miR378
I
miR148a
J
miR218


A
K14
WT
B
WT
C
Dicer fl/fl;K14Cre
D
Dicer
Relative expression level
1.2
0.8
0.4
0
WT
Dicer fl/fl;K14Cre

(A)


WT

(B)


Dicer^fl/fl;Wnt1Cre

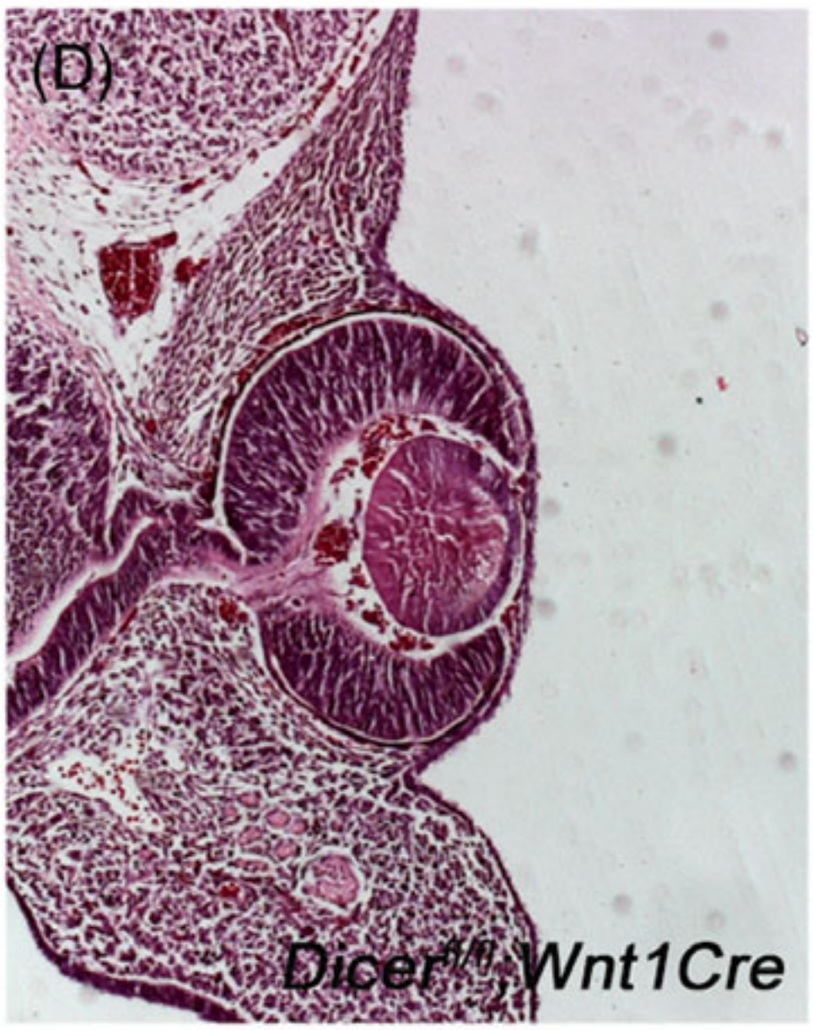
(C)


WT

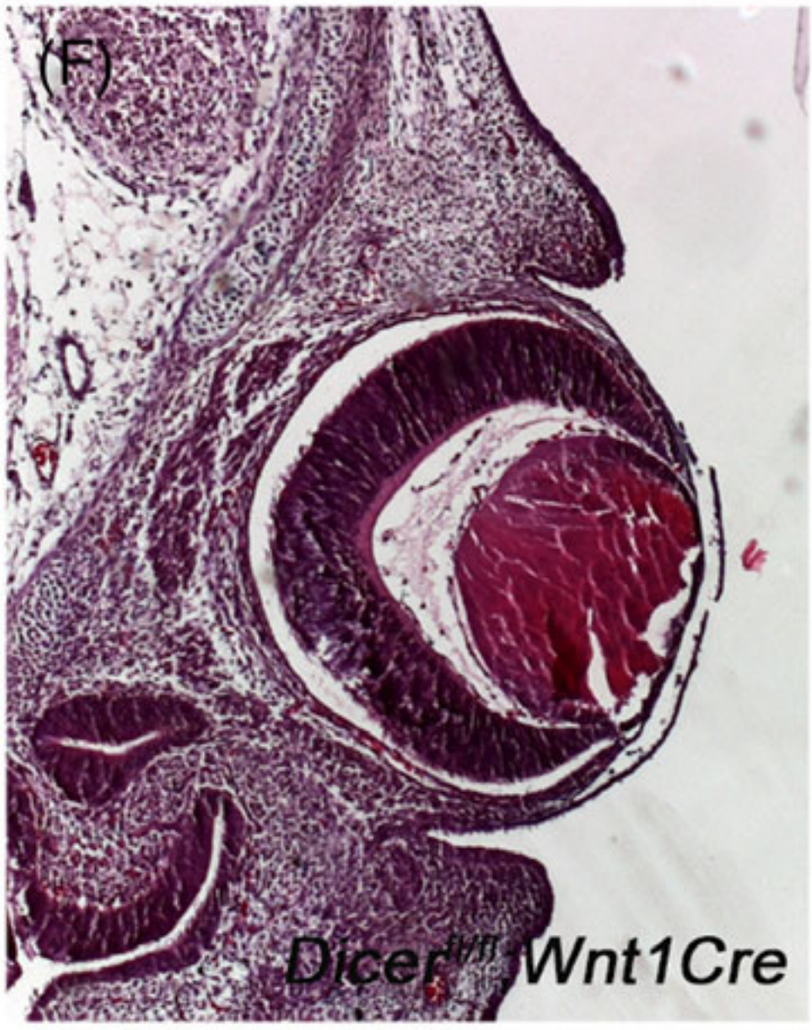
(E)


WT

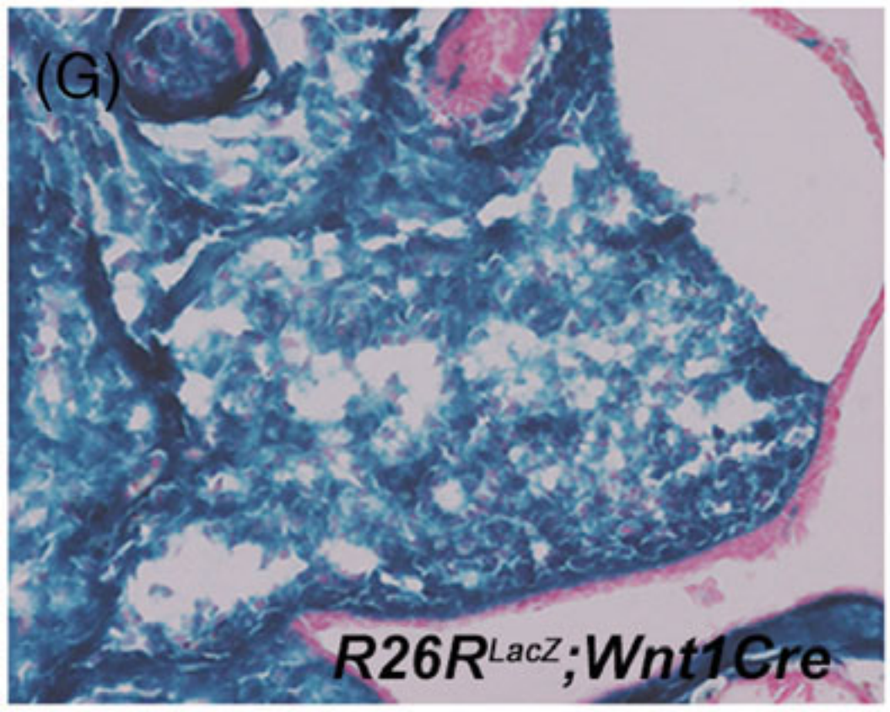
(D)


Dicer^fl/fl;Wnt1Cre

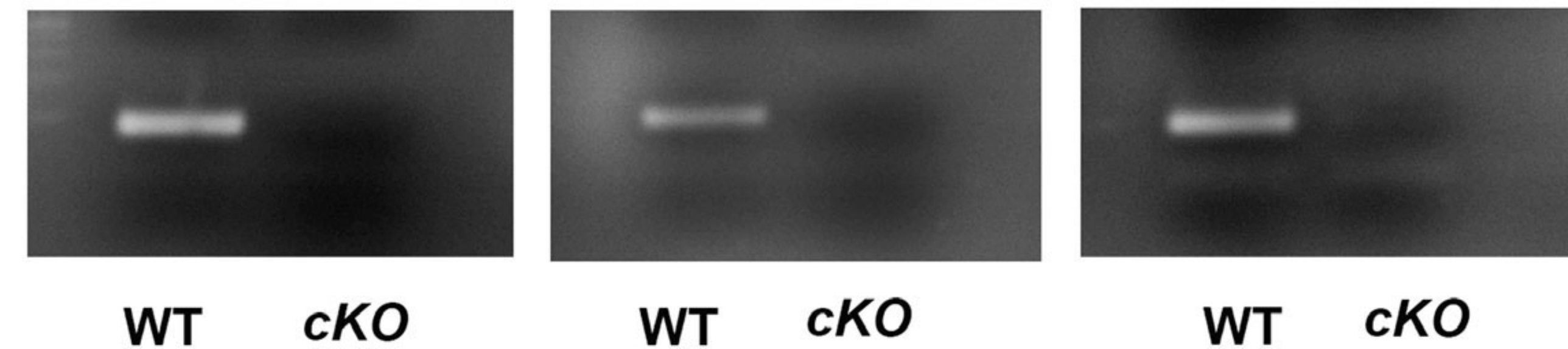
(F)


Dicer^fl/fl;Wnt1Cre

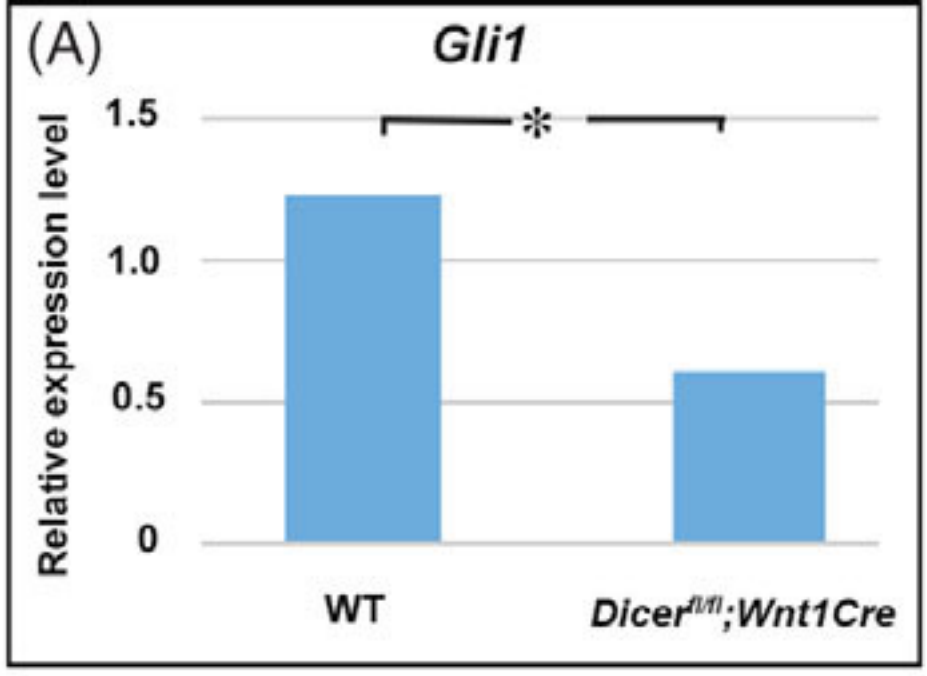
(G)

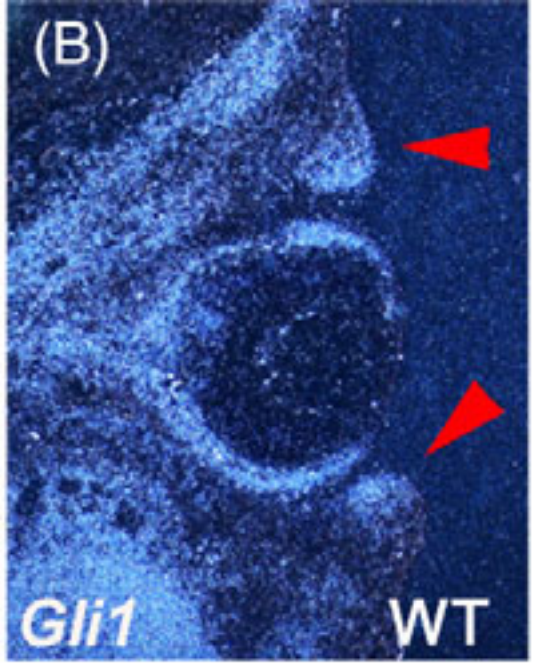

R26R^LacZ;Wnt1Cre

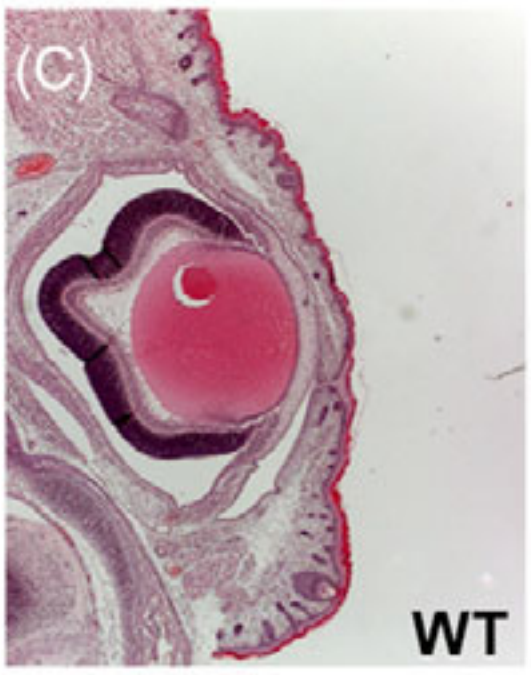
(H)

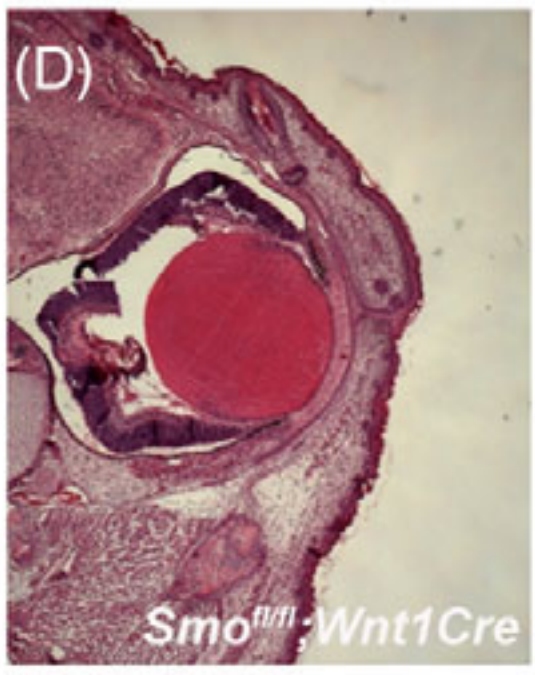

miR148a
miR218
miR378a
WT
cKO
WT
cKO
WT
cKO

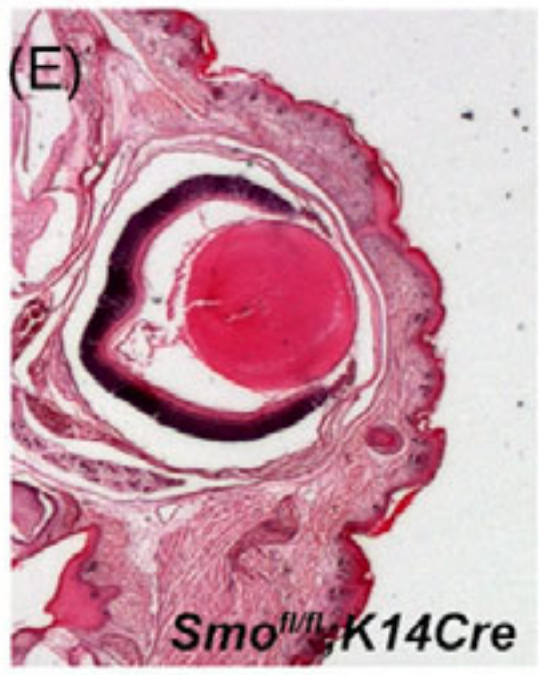
(A)
Axin2
WT
(B)
Dicer$^{fl/fl}$;Wnt1Cre
(C)
Nkd1
Relative expression level
10
5
0
*
WT
Dicer$^{fl/fl}$;Wnt1Cre
(D)
WT
(E)
Dicer$^{fl/fl}$;Wnt1Cre
(F)
Filaggrin
WT
(G)
Dicer$^{fl/fl}$;Wnt1Cre
(H)
WT
(I)
Dicer$^{fl/fl}$;Wnt1Cre
Day0
Day3
WT
(J)
(K)
(L)
Dicer KO
(M)
(N)
(O)
Dicer KO
+
DKK2
(P)
(Q)
(R)

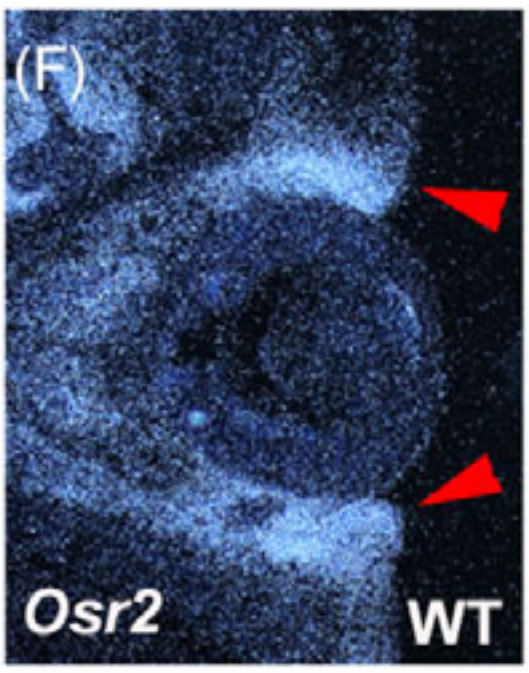


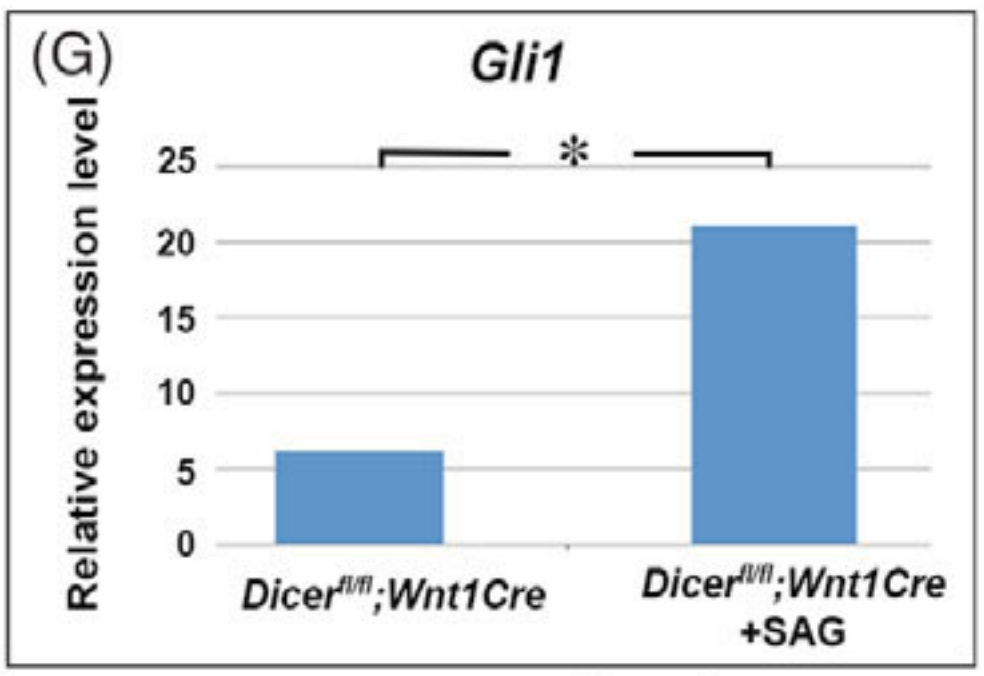








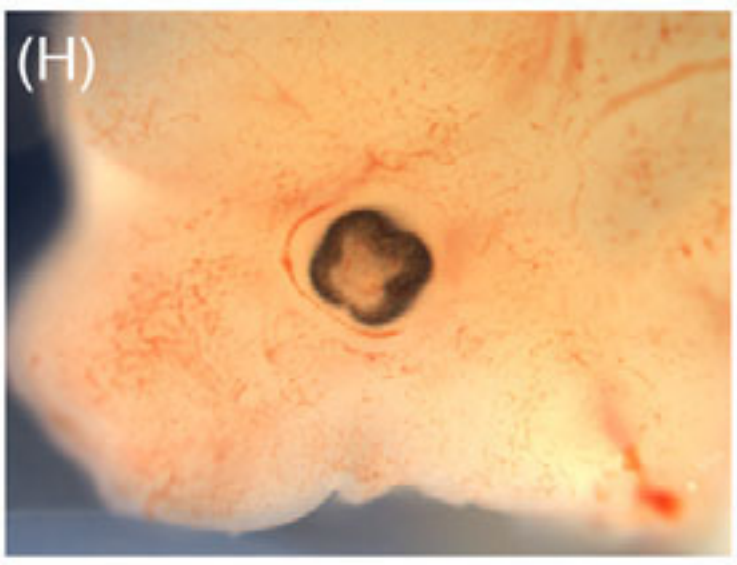


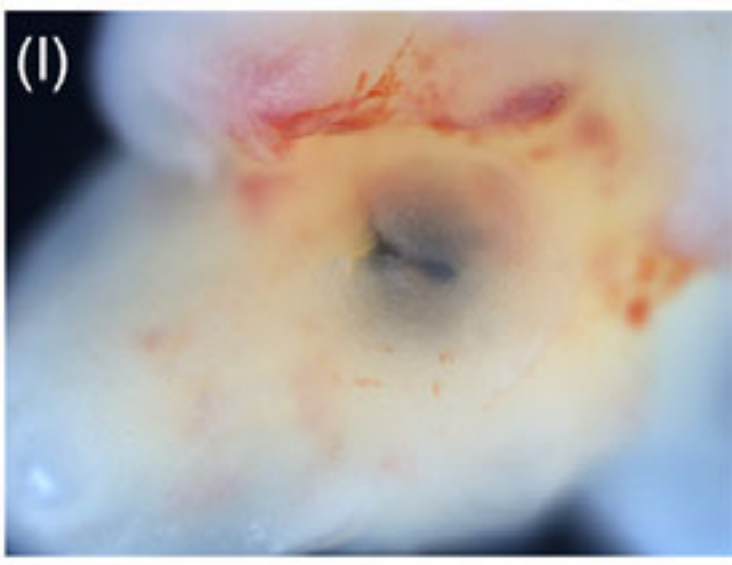


Day0

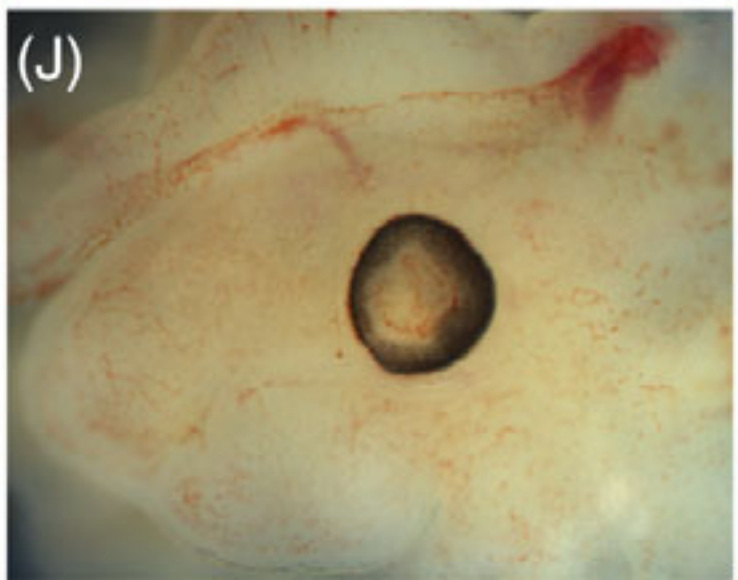
Day3

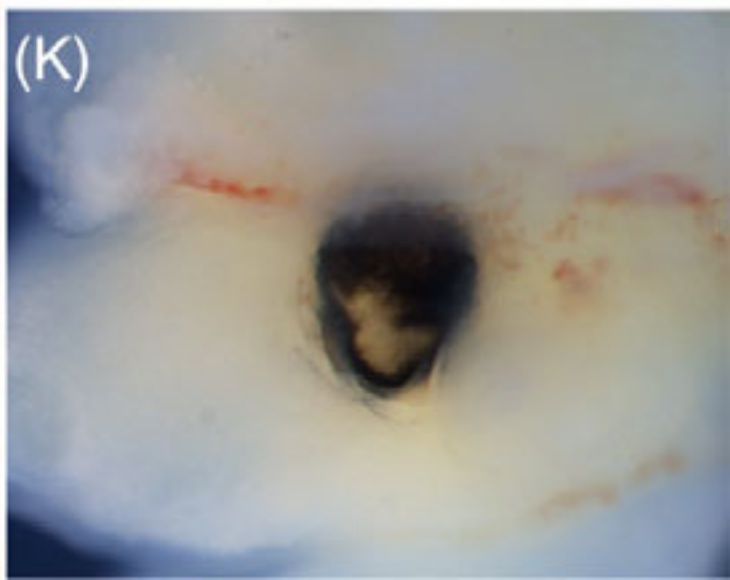
WT

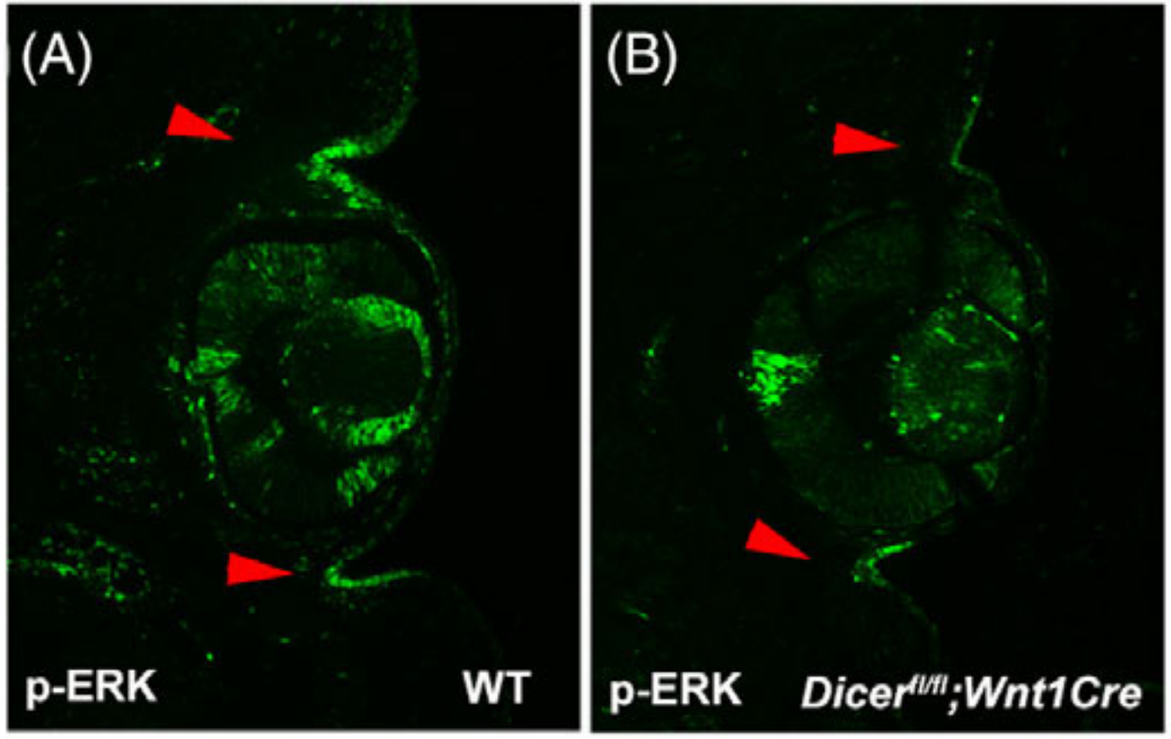


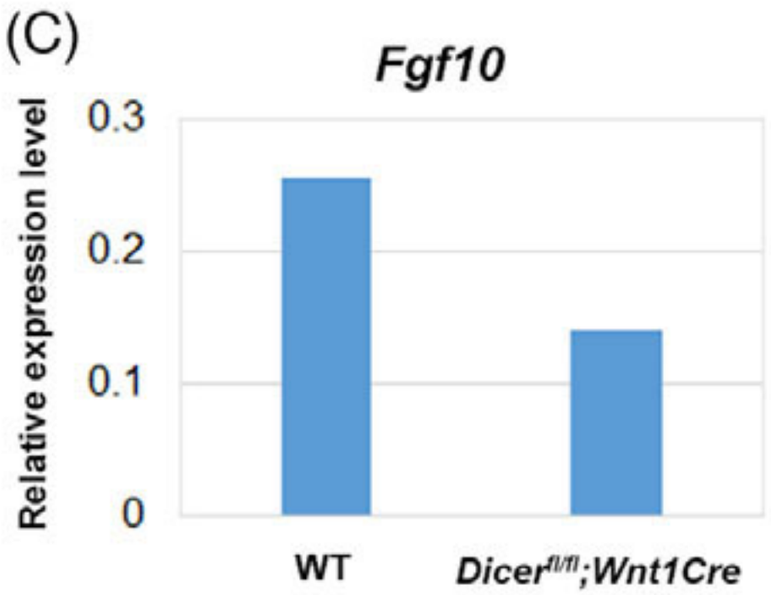


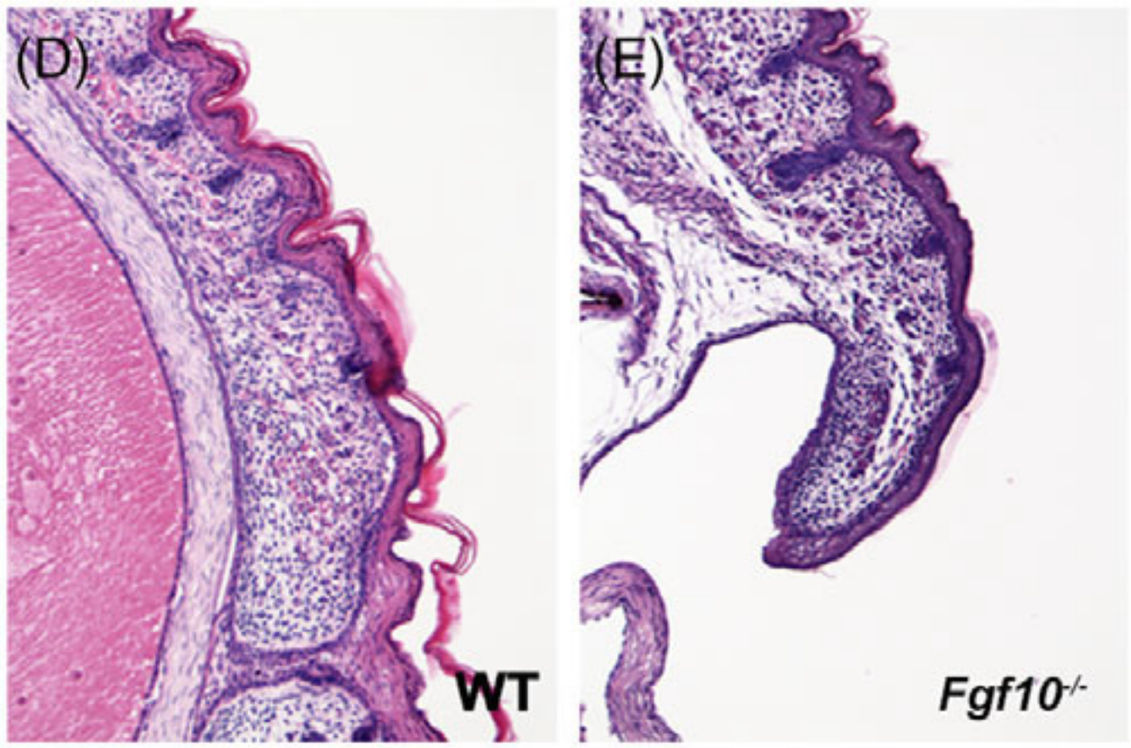
Dicer KO
+
SAG

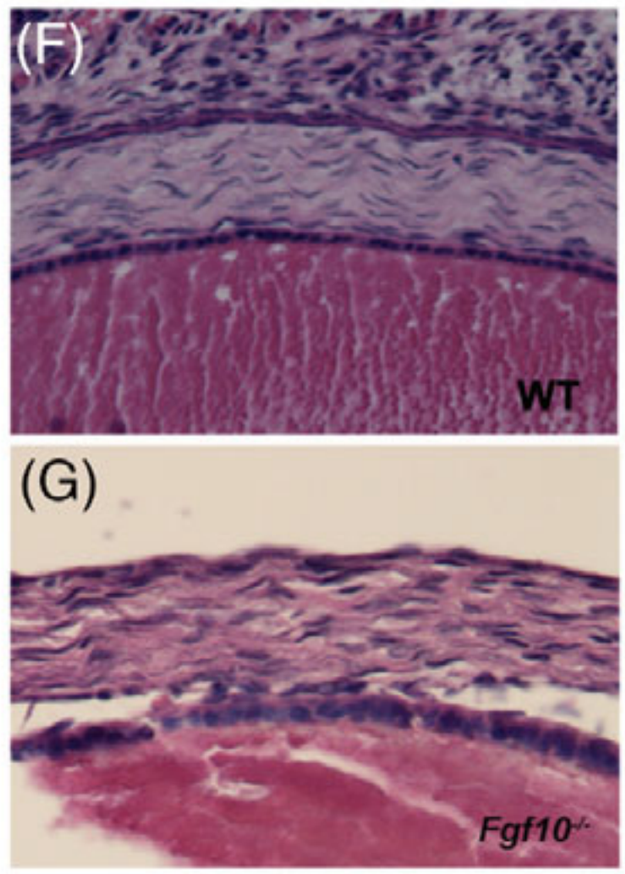


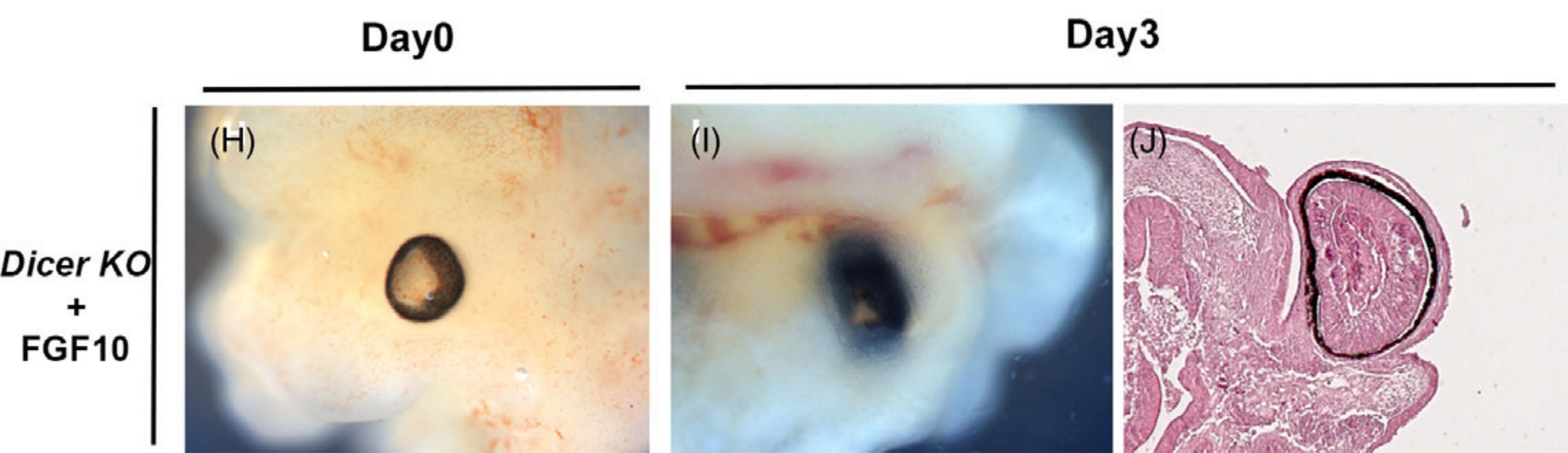



(A)
p-ERK
WT
(B)
p-ERK
Dicer^fl/fl;Wnt1Cre


(C)
Fgf10
Relative expression level
0.3
0.2
0.1
0
WT
Dicer^fl/fl;Wnt1Cre


(D)
WT
(E)
Fgf10-/-


(F)
WT
(G)
Fgf10-/-


Day0
Day3
Dicer KO
+
FGF10
(H)
(I)
(J)

(A)

p-Smad1/5/8

WT

(C)

p-Smad1/5/8

WT

(E)

p-Smad1/5/8

WT

(B)

Dicer$^{fl/fl}$*;Wnt1Cre*

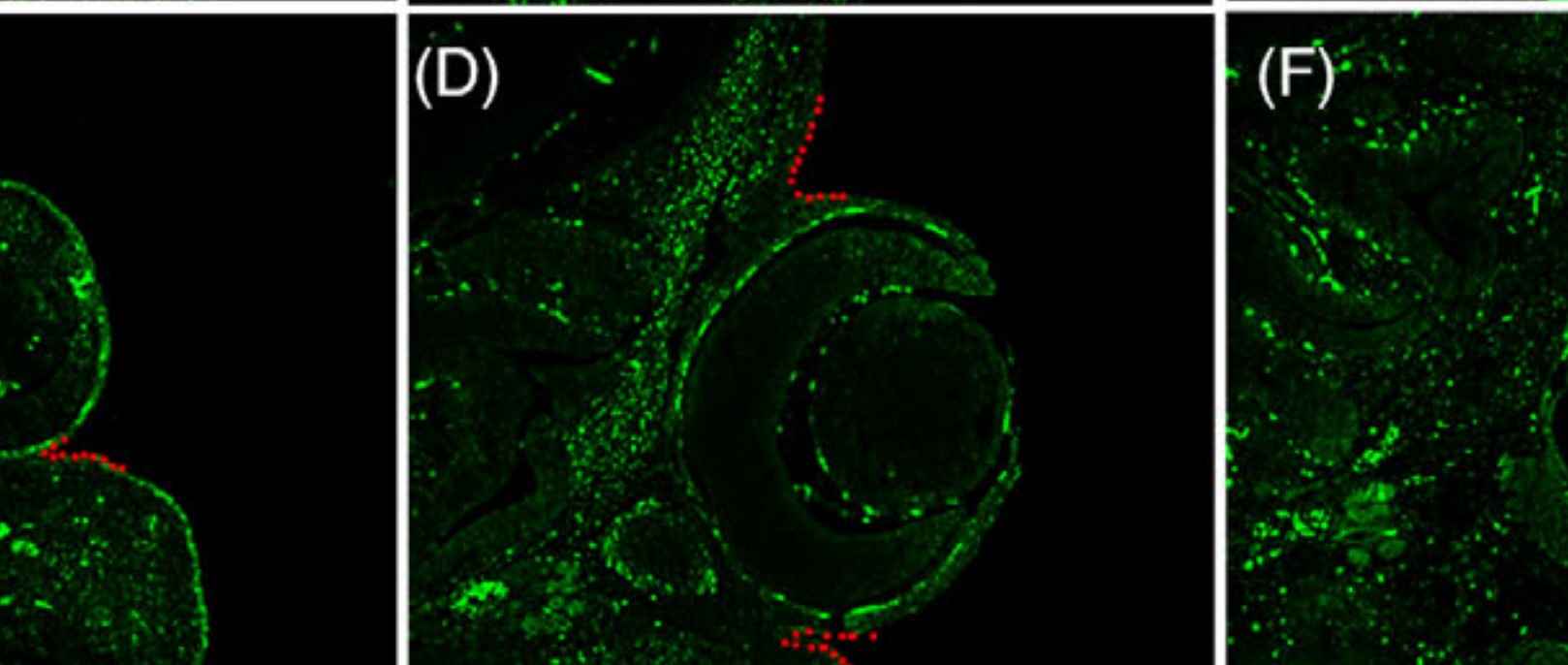
(D)

Dicer$^{fl/fl}$*;Wnt1Cre*

(F)

Dicer$^{fl/fl}$*;Wnt1Cre*

(G)



Day0

Day3

WT

(H)

(I)

Dicer KO

(J)

(K)

Dicer KO
+
BMP4

(L)

(M)